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PRINCIPAL INVESTIGATOR: Careen Tang, Ph.D.

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20057

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13. ABSTRACT (Maximum 200 Words) Among the growth factor receptors, members of the class I receptor tyrosine kinase family (ErbB) is most frequently implicated in human breast cancers. In the last report, we delineate the biological function of ErbB-4 by down-regulation of ErbB-4 expression in breast cancer cell lines with specific ErbB-4 ribozymes. To further elucidate the significance of ErbB-4 in breast cancer, we investigated the expression of ErbB-4 in primary breast carcinoma by utilizing immunohistochemical analysis with an anti-ErbB-4 monoclonal antibody on a breast cancer tissue microarray. ErbB-4 expression was found in 60% (344 of 571) of the 571 samples examined. High intense immunoreactivity of ErbB-4 was detected in 13% (74 of 571) of these primary breast tumors. Most of the staining was found in both cell membrane and cytoplasm. No expression was detected in majority of benign tumors. These results suggested that ErbB-4 might play a role in breast cancer progression. We also extend the ErbB-4 studies to ErbB-3 to explore the pathogenic role of ErbB-3 in human breast cancer cells. Two of the ErbB-3 ribozyme were generated. The catalytic activity of these ribozymes was first evaluated in an extracellular system. These ErbB-3 ribozymes can cleave ErbB-3 mRNA precisely and efficiently under physiological conditions in a cell free system. However, these ribozymes were inactive in a cellular system which indicated that the selected targeting ErbB-3 cleave sites were not accessible or the ribozyme ErbB-3 mRNA were located in different subcellular localization in these 32D/E2+E3 cells. The major challenges are still involved in studying the ability of a ribozyme to down-regulate targeted gene expression. The complexity of heterodimerization and transphosphorylation between the family receptors in the cells makes it even more difficult.				
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FOREWORD

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Caren K. Tong 7/30/00
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PART 1:

INTRODUCTION

Statements of rationale: Interest in the ErbB family of receptors arose not only because members of the family are widely expressed in mesenchymal, neuronal and epithelial cells, but also because they are implicated in the development of human adenocarcinomas (1-3). Our previous work demonstrated that *heregulin* (*HRG*), a ligand of ErbB-3 and ErbB-4, is involved in human breast cancer progression (33). In our proposal we plan to utilize hammerhead ribozymes which target HRG and its receptors (ErbB-3, ErbB-4) to interrupt their signaling. We will then study their roles in cell proliferation, hormone sensitivity and tumorigenicity. These results will provide us with important information relating to the biological significance of their expression in human breast cancer. These data will support the potential of using ribozymes as therapeutic agents for human breast cancer.

Background: The EGF receptor (EGFR) family is one group of tyrosine kinases that is frequently overexpressed in a variety of carcinomas (1-3). This class I subfamily of receptors comprises four members: HER1/EGFR (4), HER2/ErbB-2/*neu* (5-10), HER3/ErbB-3 (11, 12), and HER4/ErbB-4 (13). A number of growth factors, classified as EGF-like ligands, have been identified that can bind and stimulate the kinase activity of EGF-family receptors. EGF (14), transforming growth factor (TGF) (15), amphiregulin (AR) (16), heparin-binding EGF (HB-EGF) (17, 18), and betacellulin (BTC) (19) has been described as specific for EGFR. Several differentially spliced variants named heregulin (HRG), or a *neu* differentiation factor (NDF) (20-23), acetylcholine-receptor inducing activity (ARIA) (24), glial growth factor (GGF) (25) and gp30 (26) were initially identified as candidate *neu* ligands by their ability to induce *neu* tyrosine phosphorylation (27, 28). However, recent results demonstrate that ErbB-3 and ErbB-4 are the primary receptors for heregulin (29, 30). Activation of ErbB-2 by HRG is thought to occur through transphosphorylation resulting from heterodimerization with either ErbB-3 or ErbB-4 (31, 32). Recently, betacellulin was also shown to activate the ErbB-4 receptor and ErbB-2/ErbB-3 heterodimers in a Ba/F3 and 32D cells systems (35, 39). Most human breast cancer cells express more than one of the EGF family receptors and different combinations of receptors can heterodimerize or homodimerize. This could activate different signaling pathways and contribute to the pathogenicity and tumorigenicity of breast cancer (28, 29, 32).

Amplification and/or overexpression of EGFR and ErbB-2 are clearly important factors in neoplastic transformation of breast epitheliums (1, 2). However, ErbB-3 shows a wide range of expression in breast cancer. Overexpression of ErbB-3 is associated with the presence of lymph node metastasises, but there is no correlation with patient survival at the present time (3). Little are known about the expression or the clinical significance of ErbB-3 receptors in the diagnosis and prognosis of human breast cancer. It is therefore imperative that the role of ErbB-3 and its biological significance in breast cancer be defined. To achieve this goal, we employed ribozyme technology to disrupt ErbB-3 expression in human breast cancer cells.

In a last report, we discussed our research progress during the 7/1/98-6/30/99 in investigating the biological effects of interruption of ErbB-4 pathogenic pathways by ribozymes in human breast cancer. To delineate the biological function of ErbB-4 receptors in breast cancer, we employed a

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hammerhead ribozyme strategy to achieve down-regulation of ErbB-4 receptors in various breast cancer cell lines. We observed that down-regulation of ErbB-4 in estrogen receptor positive (ER+) cell lines (MCF-7 and T47D) resulted in a reduction of tumorigenicity both *in vitro* and *in vivo*. However, over time completely down-regulation of ErbB-4 in ER+ cell lines acquired the ability to up-regulate EGFR or ErbB-2 and progressed to a hormone-independent phenotype. These results mimic the clinical observation. Overexpression of EGFR and ErbB-2 is inversely correlated with ER. The expression of ErbB-4 was correlated with ER+ and PgR+ primary breast tumors by immunohistochemistry. These results suggested that ErbB-4 plays different roles in breast cancer progression. These results have **published in Cancer Research 59: 5315-5322, 1999 (34).**

Our data also suggested that a complex combination of regulatory mechanisms is involved in this hormone-independent phenotype. ErbB-4 expression is necessary for maintaining ER expression in breast cancer cells and is important for estrogen repression of EGFR and ErbB-2 expression. Down-regulation of ErbB-4 expression disrupts this regulation and exhibits an up-regulation of EGFR and ErbB-2 expression, and ultimately, leads to develop a more aggressive phenotype.

BODY

In this report, we will describe the progress that made during the 7/1/99-6/30/00.

Since, we observed that down-regulation of ErbB-4 reduced tumorigenicity in breast cancer cells. To further confirm our pilot studies we extend our immunohistochemical analysis of ErbB-4 expression in breast cancer tissues. We also extend the ErbB-4 studies to ErbB-3 to explore the pathogenic role of ErbB-3 in human breast cancer cells, using an approach similar to that in the ErbB-4 studies. Our goal is to generate the ErbB-3 ribozymes and study the effects of down-modulation of endogenous ErbB-3 levels in breast cancer cells *in vitro* and *in vivo*.

Results:

Clinical Significance of ErbB-4 Expression in Breast Cancer Patients.

We investigated the expression of ErbB-4 in primary breast carcinoma, using immunohistochemical analysis with an anti-ErbB-4 monoclonal antibody. Dr. Olli Kallioniemi (NIH) has kindly provided us breast cancer tissue microarray, which allow us to examine 571 different cases of primary invasive breast cancer in a single slide under uniform immunohistochemical conditions. No expression was detected in majority of benign tumors. ErbB-4 expression was found in 60% (344 of 571) of the 571 samples examined. High intense immunoreactivity of ErbB-4 was detected in 13% (74 of 571) of these primary breast tumors (Figure 1). Most of the staining was found in both cell membrane and cytoplasm. Table 1 summarized the immunohistochemical results for ErbB-4 expression in primary invasive breast cancer microarray.

Table 1

ErbB-4 Expression Levels	% of expression
High expression (+++)	13% (74/571)
Intermediate/low expression (++/+)	47% (270/571)
No expression	40% (220/571)

Design and Generation of ErbB-3 ribozyme:

To investigate the biological significance of ErbB-3 in human breast cancer cells, we used molecular targeting of the ErbB-3 mRNA by ribozymes. We used GCG Package Database program to select the ribozyme sequence. First, we used this program to predict the optimal and sub-optimal secondary structure of ErbB-3 mRNA using the most recent energy minimization method by Zuker. Secondly, we selected ribozyme target sites in the open loop regions with the GUX cleavage site. Thirdly, we then used the same program to predict the secondary structure of the selected regions to see whether or not these sequences are able to fold into a typical hammer headed ribozyme three stem loop structure. Finally, we tested the selected ribozyme sequences for specificity against other known human genes in the GenBank database.

Two ribozymes targeted to specific sites of the ErbB-3 mRNA open reading frame were selected. The following are the sequences of the targeted ErbB-3 mRNA sites. ErbB-3Rz198 targets at 5' CUC UGC GGA GUC AUG AGG GCG 3', and ErbB-3Rz528 targets at 5' GGG ACC CAG GUC UAC GAU GGG 3' under line indicates the cleavage sites.

These ribozymes were modeled on the previously described hammerhead structure (35), derived and minimized to the catalytic center portion of 22 nucleotides. We generated two of the ribozymes targeted in the ErbB-3 mRNA (See figure 2).

Demonstration of ErbB-3 ribozyme catalytic activity in a cell free system

The catalytic activity of these ribozymes was first evaluated in an extracellular system. These ErbB-3 ribozymes can cleave ErbB-3 mRNA precisely and efficiently under physiological conditions in a cell free system. Cleavage was specific as the actual sizes of the cleaved fragments correspond to the expected sizes if cleavage were occurred immediately 3' to the GUN sequence. As an efficacy control, catalytically inactive mutant ribozymes were engineered. The point mutation of G to A in the catalytic domain of either Rz198 or Rz528 results in a loss of catalytic activity as predicted by the mutational studies of McCall et al. (35). We also tested the specificity of these ErbB-3 ribozymes by using ErbB-3 mRNA as a substrate. Figure 2 illustrates the catalytic activity of these ErbB-3 ribozymes (Figure 3). No cleavage was observed by point mutation of these ribozymes (Figure 3). These results indicate that all three of the GUN sequences chosen in the ErbB-3 mRNA are accessible to ribozyme mediated cleavage in an extracellular system.

An intracellular model system for studying the specificity and efficacy of these ErbB-3 ribozymes

We next investigated the catalytic activity of these ribozymes intracellularly. Although the ribozyme sensitivity in an extracellular system can be correlated with the predicted secondary structure of the target RNA, the intracellular susceptibility of the target RNAs to ribozymes cannot necessarily be correlated with their predicted secondary structure. This is usually due to stability, accessibility and subcellular localization of the ribozyme species *in vivo*. There are major challenges involved in studying the ability of a ribozyme to down-regulate endogenous gene expression. The complexity of heterodimerization and transphosphorylation between the family receptors in breast cancer cells makes it difficult to determine the specificity of these ErbB-3 ribozymes. Furthermore,

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the goal of these ribozymes is to interrupt gene expression. If ErbB-3 is one of the critical factors involved in cell proliferation, down-regulation of this gene may be lethal to the cells. Thus, an ideal system for screening the intracellular enzymatic activity of these ribozymes requires the following criteria. 1) Expression of high levels of ErbB-3 receptor. 2) No expression of other EGF family receptors. 3) Non-lethality of ErbB-3 ribozyme introduction. 4) Easy detection of ribozyme activity by bio-assay. The 32D cell system meets these requirements, and therefore was used as a model system to examine the intracellular efficacy and specificity of these ribozymes. 32D cells are a murine hematopoietic IL3-dependent cell line that does not express detectable levels of endogenous EGF-family receptors. Studies have shown that IL-3-dependence can be abrogated by introduction of foreign growth factor receptor genes followed by stimulation with the appropriate growth factor (36, 37). However, ErbB-3 is a kinase defected receptor, it requires ErbB-2 heterodimers to trans-activate. This approach exploits a unique property of this model system. If HRG can induce ErbB-3/ErbB-2 transfected cells to bypass the IL3-dependent pathway, we can then use a simple growth assay to determine the biological function of these ribozymes intracellularly.

Biological function of EGF family receptors in 32D cells: 32D cell derivatives were established that ectopically express the EGF family receptors singly and in pairwise combinations (36). The resultant stably transfected cells were designated as 32D/E1, 32D/E2, 32D/E3, 32D/E2+E3 and 32D/E4. E1, E2, E3 and E4 refer to EGFR, ErbB-2, ErbB-3 and ErbB-4 receptors. The expression of the receptors in the 32D cells was confirmed by western blotting or immunoprecipitation followed by western blotting (data not shown). No detectable levels of endogenous EGF family receptor expression were found in the parental 32D cells. The 32D transfected cells express high levels of the corresponding receptors. In the absence of cognate ligands, all of the 32D transfected cells remained dependent on IL-3 for survival. We then tested whether HRG was able to induce the IL-3-independent survival or proliferation of these 32D transfected cells. The untransfected parental cells were not stimulated by HRG. Cells transfected with ErbB-4 or co-expressing ErbB-2 and ErbB-3 respond to HRG stimulation, bypassing the IL3-dependent pathway, but cells transfected with ErbB-2 or ErbB-3 alone do not respond to HRG stimulation (36, 37). Regulation of tyrosine phosphorylation of each receptor by HRG was evaluated by immunoprecipitating the corresponding receptors and immunoblotting with antiphosphotyrosine. 32D/E2 cells demonstrated significant endogenous phosphorylation of ErbB-2, but receptor phosphorylation was not elevated in the presence of HRG. No phosphorylation was observed in the presence or absence of HRG in 32D/E3 cells. In 32D/E2+E3 cells, we observed a high basal level of phosphorylated ErbB-3, and were able to further induce phosphorylation by HRG (37). Thus, this is an ideal system to study the specificity and efficacy of the ribozymes targeting the ErbB- family receptors. This system can be turned on and off by IL-3 or the appropriate ligands. Therefore, a simple growth assay will be able to define the intracellular enzymatic activity of the ribozymes.

Determination of ErbB-3 ribozyme catalytic activity in 32D cells:

We constructed two of these ErbB-3 ribozymes in a mammalian expression vector under a CMV promoter control. We then transfected the ErbB-3 Rz into 32D/E2+E3 cells. We hypothesized that the functional ribozymes would down-regulate ErbB-3 expression and thereby reduce or abolish

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the HRG induced, IL-3-independent survival or proliferation. We performed a growth assay in the presence and absence of HRG using these ErbB-3 Rz transfected cells. We did not observe any effect on the HRG induced IL3-independent phenotype. We then evaluate the intracellular enzymatic cleavage activity of ErbB-3 ribozymes by examining their target mRNA levels with northern blot analysis. As expected, no effect on ErbB-3 mRNA levels was observed. These data suggest that although these ribozymes were able to cleave ErbB-3 mRNA specifically in a cell free system. These ribozymes were inactive in a cellular system which indicated that the selected targeting ErbB-3 cleave sites were not accessible or the ribozyme ErbB-3 mRNA were located in different subcellular localization in these 32D/E2+E3 cells.

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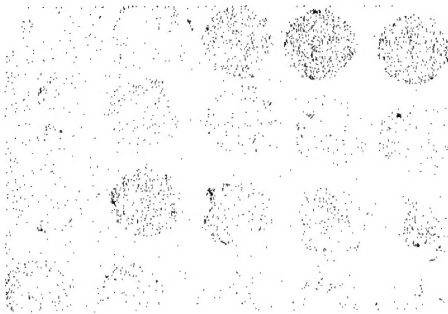
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ILLUSTRATION

A.



B.

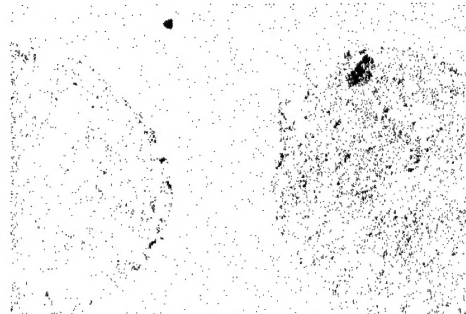
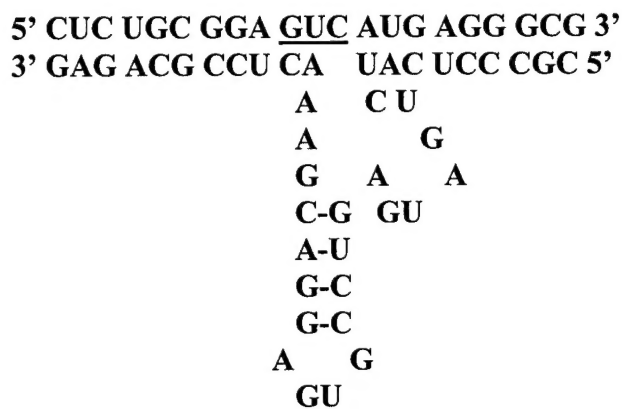


Figure 1. Illustrates the immunohistochemical analysis on a breast cancer tissue microarray with anti-ErbB-4 antibody and counterstained with hematoxylin for viewing negative stained cells (Blue). ErbB-4 positive stained cells shows in brown color. A and B illustrate the breast tissue microarray under different magnification.

ILLUSTRATION

ErbB-3 Rz 198



ErbB-3 Rz 528

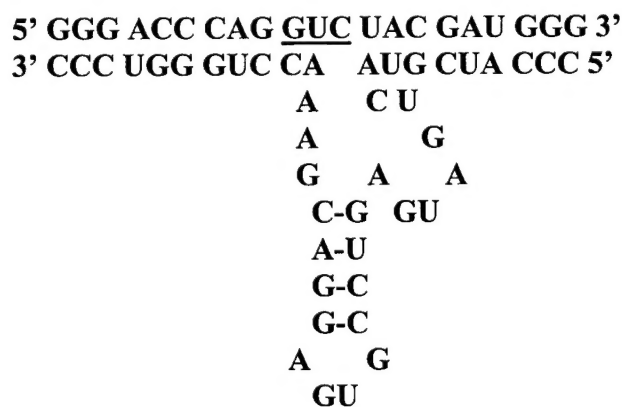


Figure 2. Illustration of hammer head structure of ErbB-3 ribozymes. Underline GUC are the targeted cleavage sites in the ErbB-3 mRNA.

ILLUSTRATION

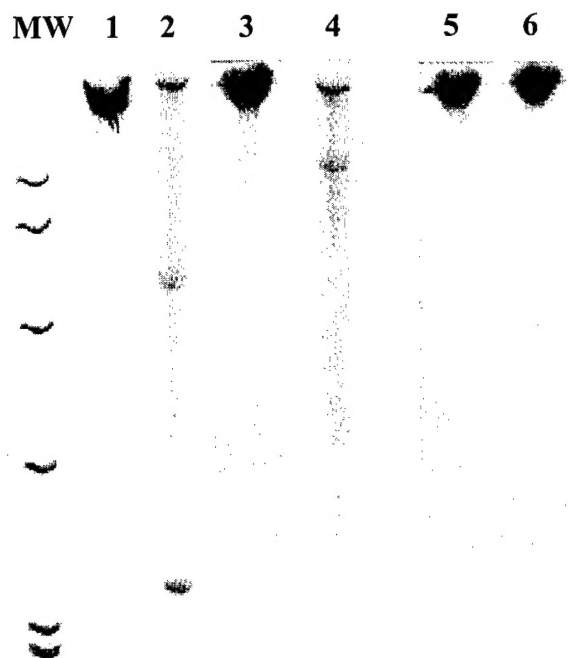


Figure 3. Catalytic activity of ErbB-3 ribozymes in a cell free system. MW: Molecular weight. Lane 1. Full length of ErbB-3 mRNA. Lane 2. ErbB-3 Rz 528 cleavage products. Lane 2. Full length of ErbB-3 mRNA. Lane 4. ErbB-3 Rz 198 cleavage products. Lane 5 and 6 point mutation of ErbB-3 Rz 198 and ErbB-3 Rz 528 do not cleave ErbB-3 mRNA.